An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

15S669 - Small-molecule Modulators of Oomycete Calcium Channels

Final Report

This project was funded under the Department of Agriculture, Food and the Marine Competitive Funding Programme.

SUMMARY

Phytophthora infestans is a member of a group of fungus-like organisms called oomycetes, and is the cause of late blight of potatoes. The central aim of this project was to develop new classes of crop-protective fungicides, based on their interaction with a particular type of molecular switch, called PKD-RR calcium channels, that are only present in oomycetes and not in other organisms such as animals and plants. This is important, because Ca2+ is a major regulator of life and death in cells, so altering the function of the Ca2+ channel in P.infestans using new fungicides could selectively kill this pest. We attempted to screen for new molecules that interact with the P.infestans channel by transfecting mammalian cells with the gene encoding it, as it is much easier to measure changes in Ca2+ in these rather than in oomycetes. However, this approach was unsuccessful, because although the transfected mammalian cells expressed the PKD-RR channel protein, it was not produced in a functional state. As an alternative, we directly screened the effects of a range of small molecules on P.infestans growth and zoospore release. These molecules had some structural features of cinnamaldehyde (CA), a plant molecule that has anti-oomycyte properties and which might interact with certain Ca2+ channels. Several of the molecules that we tested potently inhibited the mycelial growth of P.infestans and enhanced release of the motile, zoospore stage (both at low micromolar concentrations) . These molecules had little effect on the viability of mammalian cells. Towards the end of the project, we investigated the commercial potential of these anti-oomycete molecules via a gatekeeper agreement with the agrochemical company, Syngenta. Although, the company decided not to explore these molecules further, we aim to further refine and develop them as a workpackage in a H2020 application, led by Dr. Doyle-Prestwich.

KEYWORDS

Potato blight, fungicide, calcium channel

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Section 1 - Research Approach & Results

Start Date

01 January 2017

End Date

31 March 2019

Research Programme

Research Stimulus Fund

TRL Scale

TRL 4: Technology validated in lab

NRPE Priority area

Sustainable Food Production and Processing

Total DAFM Award

€195,086.00

Total Project Expenditure

€189,094.73

Rationale for undertaking the Research

Late blight, caused by P.infestans, is a devastating pathogen of potatoes and tomatoes, costing in excess of $10 billion globally per annum in terms of crop losses and application of pesticides. Other members of the phylum Oomycetes cause significant losses in the agriculture, forestry and fisheries sectors. The key crop protective measure is spraying of crops with fungicides. However the use of mancozeb, the main fungicide that is protective against P.infestans, is currently under review within EU. Furthermore, some naturally occurring strains of P.infestans display resistance to recently developed oxathiapiprolin fungicides. Consequently, there is a demand for new, selective antiblight fungicides. Given that the essential oil component cinnamaldehyde (CA) is reported to kill oomycytes and to raise intracellular Ca2+ concentrations in other Phytophthora species, we aimed to develop novel anti-blight fungicides based on the structure of this molecule.

Methodology

The following methodologies were employed:

1) Mammalian cells were transfected with a plasmid encoding a P.infestans PKD-RR Ca2+ channel, tagged with green fluorescent protein(GFP), to enable confirmation of transfection. Several different transfection strategies were attempted, eg. transient versus stable transfections; transfection of different cell types.
2) The cytoplasmic Ca2+ concentrations in PKD-RR-GFP transfected cells were determined using FLIPR technology, both at rest and inresponse to CA and related molecules.
3) Organic chemistry approaches were used to synthesize and characterize a range of molecules that share some of the structural featuresof CA. Some of these structures were synthesized on an ellipticine backbone.
4) These novel molecules and a pre-existing library of other compounds were screened for their ability to inhibit the mycelial growth ofP.infestans cultures. This was achieved by incorporating known concentrations of the molecules into Rye A agar plates. Plugs of P.infestans cultures were then transferred to these plates, and the diameters of the resulting colonies measured at various time-points. CA and mancozeb were used as positive controls in these experiments.
5) The same molecules were screened for their effects on P.infestans zoospore release. Cultures were grown on Rye B agar containing the molecules, until they developed sporangia (approximately 2 weeks). Sporangia were then harvested, and the number containing zoospores divided by the total was counted by light microscopy.
6) P.infestans was cultured on Rye B agar plates until sporangiogenesis had occurred. Zoospores were released from sporangia byincubating the plates with ice-cold Petri's medium for 2 hours. Zoospore motility was monitored, in the presence or absence of candidate fungicides, using videomicroscopy. The characteristics of motility (velocity, total distance, euclidean distance, directionality) were determined using ImageJ software.
7) The effects of these molecules on micro-propagated potato plants and on mammalian cell viability were also evaluated.

Project Results

1) Mammalian cells were successfully transfected with the PKD-RR-GFP cDNA, as assessed using fluorescent microscopy (to detect GFP) and by Western blotting (using an antibody recognising the GFP tag, or a custom antibody against PKD-RR protein itself).
2) However, the expressed PKD-RR-GFP protein probably did not form functional Ca2+ channels, as judged by there being no significantdifference in resting cytoplasmic Ca2+ between PKD-RR-GFP and GFP-only expressing cells; nor were there any detectable changes in Ca2+ in response to CA. All cells demonstrated increases in Ca2+ in response to ATP, which activates a G-protein coupled receptor coupled to IP3 production and Ca2+ release from intracellular stores (a positive control). We conclude that the PKD-RR-GFP channel was not functional when heterologously expressed in mammalian cells, possibly because it was lacking a factor present in oomycetes, but not in mammals, eg. a specific channel accessory protein.
3) Organic chemical synthesis strategies were developed for the production of ellipticine scaffolds, bearing moieties resembling thosefound on CA. The structures of these new molecules were verified by mass spectroscopy, infra-red spectroscopy and NMR. Sufficient quantities and purities of these molecules were achieved to enable testing of their effects on P.infestans, potato plant and mammalian cell biology.

4) Of 32 molecules tested, seven caused significant inhibition of P.infestans mycelial growth at all time-points tested (5, 9 or 13 days). The effects of one of these lead molecules, "MM74", were determined in greater detail. After 13 days, at a concentration of 10 micromolar, MM74 reduced growth to 6% of control values, compared with 77% for 100 micromolar mancozeb. From concentration-growth inhibition data, MM74 had a half-maximal effective concentration of 6 micromolar, compared with 275 micromolar for CA.
5) Similar effects of these molecules were found in terms of zoospore release, with the seven molecules that inhibited mycelial growth alsopromoting sporangial emptying, at a concentration of 10 micromolar.
6) At a concentration of 25 micromolar, only one of these molecules ("RAK10") had any effect on zoospore motility. RAK10 decreased zoospore velocity to a similar degree to 100 micromolar mancozeb. RAK10 might represent a lead molecule for the development of fungicides decreasing the spread of blight, by inhibiting zoospore motility.
7) None of the seven lead molecules identified had any large effect on the viability of a mammalian cell-line, as assessed using the XTT assay (a modified MTT assay, for mitochondrial activity). Experiments on micro-propagated potato plants indicate that MM74 might be protective against P.infestans infections.

Overall, these results demonstrate the development of a new class of anti-blight fungicides. They are exemplified by molecule MM74, which reduces mycelial growth to 6% of control values after 13 days; enhances zoospore release; has minimal cytotoxicity in mammalian cells; and displays protective effects in potato plants against P.infestans infection.

Section 2 - Research Outputs

Summary of Benefits / Improvements of Project Findings

The research funded by this grant has provided the following benefits:
1) Formation of an interdisciplinary network of scientists that will persist into the future.
2) Training of a research assistant, a masters student and a postdoctoral scientist to a high standard. These highly skilled individuals will potentially enhance Irish industry, academia and agriculture.
3) Published and planned dissemination of new knowledge in scientific journals. This knowledge will be of benefit to other stakeholders interested in controlling P.infestans and related pathogens, including scientists and researchers in the agrochemical industry.
4) Development of new classes of fungicides, that impair P.infestans growth and biology. Although these fungicides are not market-ready, it is expected that increased knowledge of their mode(s) of action will lead to the development of commercial product(s). This will be of benefit to potato growers, sprayers, the food industry and the consumer.
5) The knowledge generated in this project will be used to leverage further research funding. Although a recent application for this purpose was rejected by DAFM, a similar project forms part of a workpackage in an H2020 application led by Dr. Barbara Doyle-Prestwich (a collaborator on the current project) for a project called "Constructing Holistic Innovative Potato Protection Strategies (CHIPPS)", submitted in January 2020. If successful, this H2020 project will have multiple benefits for Irish stakeholders.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
Post Doctorates	0	1	1
MSc Students	0	1	1
Research Technicians/ Assistants	0	1	1

Summary of Academic Outputs

Research Outputs	Total Number	Details
Peer Reviewed Conference Papers	1	Poster presentation at the International Association for Plant Biotechnology conference, Dublin: "L Zheng, B Doyle-Prestwich, F McCarthy, T Zhang, J Mackrill (2018) Investigation of PKDRR Channel Proteins in Phytophthora Infestans. IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY-PLANT 54, S123-S123"

Intellectual Property

This research is currently protected by an Invention Disclosure Form held by the Technology Transfer Office (TTO) at UCC (Case Number: IDF-19-026). We attempted to exploit several avenues to drive the commercialization of the work, including contacts with Enterprise Ireland (represented by Dr. Claire Walsh, December 2018) and development of a gatekeeper agreement with the agrochemical company Syngenta (brokered by Dr. Eleanor Cornish, TTO, UCC). After review of our data, Syngenta declined to pursue this commercialization further, mainly because of the potential DNA intercalation and toxicity properties of the lead molecules. Consequently, we are aiming to disseminate our work through scientific publications.

Summary of other Project Outputs

Project Outputs	Details	Total No.
New Industry Developed Collaborations	Please see information on Intellectual Property. Syngenta have indicated that they are willing to collaborate with us in future.	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Industry	Potential future collaboration with agrochemical partner.

Dissemination Activities

Activity	Details
Workshops at which results	Please see information on conference presentation were presented

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.	Development of new classes of anti-blight fungicides
Identify any knowledge transfer activities executed within the project.	Gatekeeper agreement with Syngenta, December 2018
List any impacts resulting from the knowledge transferred during the project.	Potential future collaborations with Syngenta

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding Resource	Funding €	Summary
EU R&I programmes	€0.00	Please see "Future Strategies"

Future Strategies

Given that the lead molecules we developed are unlikely to have any direct commercial value, owing to their potential cytotoxicity, we will disseminate this knowledge in scientific journals. A key to future developments of our work will be identification of the molecular target(s) of the fungicides developed in the course of this project, since they are highly effective at inhibiting P.infestans mycelial growth. We submitted a grant application to pursue this work to DAFM, but the proposal was rejected in September 2019. As an alternative, this project now forms a workpackage in an H2020 application made by Dr. Doyle-Prestwich, called "Constructing Holistic Innovative Potato Protection Strategies (CHIPPS)". This application was submitted in January 2020 and includes all of the principal investigators on the current project.

Project Publications

We are aiming to submit three research papers to a special issue of the journal Pathogens (ISSN 2076-0817, IF 3.405) , entitled "Biology and Pathology of Phytophthora infestans". The deadline for submission of these papers is 31st May 2020, with Open Access charges of CHF1300 (approx. €1225) per article. Payment of these charges will depend on the availability of overhead contributions from DAFM. Provisional details of first drafts are:

1) Zheng, L, Doyle-Prestwich, B and Mackrill, JJ. Characterization of a family of putative channel proteins from Phytophora infestans. (approx. 6 journal pages).

2) Kehoe, R, McCarthy, FO, Zheng, L, Doyle-Prestwich, B, Mackrill, JJ. Inhibitory properties of aldehydes and related compounds against Phytophthora infestans – identification of a new lead. (approx. 8 journal pages).

3) Mackrill, JJ, Kehoe, R, R, Zheng, L, Doyle-Prestwich, B, McCarthy, FO. Synthesis and evaluation of novel ellipticines and derivatives as inhibitors of Phytophthora infestans growth (approx. 8 journal pages).